PATENT
Attorney Docket No. **KNAUTHE-08178**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Jürgen Hescheler *et al.*		
Serial No.:	10/084,960	Group No.:	1632
Filed:	02/28/02	Examiner:	Woitach
Entitled:	**Fluorescent proteins as cell-type specific reporters**		

Declaration of Jürgen Hescheler

Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated: 2-4-04 By: [signature]

I, Dr. Jürgen Hescheler, state as follows:

1. My present position is: Professor and Director at the Institute for Neurophysiology of the University of Cologne.

2. I have reviewed the above captioned patent application, of which I am an inventor, the Office Action mailed August 29, 2001 in the parent case, and the cited references.

3. In the parent application, the Examiner rejected the Claims as obvious over Zernicka-Goetz et al. I note that the promoter used in Zernicka-Goetz et al. is a cdc2 promoter. This promoter is expressed in undifferentiated, proliferating stem cells. In this respect, the Examiner's attention is directed to page 1135, column 2, which teaches that the CDC2 promoter is active in proliferating, undifferentiated cells, and inactive in ES cells that "exit the cell cycle and differentiate in culture."

4. The fact that a GFP gene can be used as reporter of expression from a development-dependent promoter that is substantially inactive in undifferentiated stem cells is not reasonably

expected from the prior art. In this respect, I direct the Examiner's attention to Raz et al., Developmental Biology 203:290-294 (1998), paragraph bridging the left and right columns of p. 290 (attached as Tab A to the Declaration). The authors state the following:

> While GFP provides an excellent noninvasive method for monitoring gene expression, the production of signal by this method is noncatalytic. For this reason, the number of molecules required to overcome the autofluoresence background is relatively high. It was estimated that 10^5-10^6 GFP molecules per cell are required to visualize the protein when it is evenly distributed in the cytosol (Niswender et al., 1995). This low level of sensitivity can result in a time delay between onset of gene expression and the ability to detect accumulated green fluorescent protein (Davis et al., 1995), especially when weak and tightly regulated promoters are used.

This reference demonstrates that the art recognized that GFP may not be a suitable reporter for all situations, especially when a weak and tightly regulated promoter is utilized. Thus, a person of skill in the art would not conclude that GFP would necessarily work in conjunction with the developmentally regulated promoters that are substantially inactive in undifferentiated stem cells. Instead, the fact that GFP could be used with such promoters had to be determined empirically.

5. With respect to Ikawa et al., I note that they describe the use of GFP under the control of the ubiquitously expressed chicken beta-actin promoter. This work has little relevance to the present invention which utilizes development dependent promoters that are substantially inactive in undifferentiated embryonic stem cells to report gene expression in embryoid bodies. It was never envisaged by Ikawa et al. that their GFP system, or something similar to it, could be used in embryonic stem cells. In this respect, I attach as Tab B to this Declaration a printout of an e-mail of 1996 from Dr. Masaru Okabe, head of the Research Institute for Microbial Diseases and senior author of the Ikawa et al. publication, to Drs. Bernd Fleischmann and Eugene Kolossov, who are my co-workers. In his e-mail, Dr. Okabe indicated that h-x-GFP and pCX-GFP are "basically the same to pCX-h-x-GFP" stating that "Our construct may not work nicely in ES cells from our experience. In order to check the high expression of h-x-GFP you had better use other cell lines." Hence, even the authors of Ikawa et al., i.e. those who knew best about GFP in mammalian cells, were of the opinion that GFP is not a suitable marker for ES cells. For the information of the Examiner, I would like to add that the abbreviation h-x was used by Dr. Okabe for EGFP (enhanced green fluorescent protein), which was not published at that time.

PATENT
Attorney Docket No. **KNAUTHE-08178**

6. As of the filing date of the priority document, it was known that stem cells from many different mammalian species could be isolated and used to form embryoid bodies and/or transgenic animals. In this regard, the Examiner's attention is directed to the following table summarizing some of the published literature:

Reference	Species	Date	Embryoid body	Animal
U.S. Pat. No. 5,994,619	Pig, cow	12/16/96		Yes
U.S. Pat. No. 6,200,806	Primate	1/18/96	Yes	
Wheeler, Development and validation of swine embryonic stem cells: a review, Repro. Fertil. Dev. 6(5):563-8	Pig	1994	Yes	
Sims and First, Production of calves by transfer of nuclei from cultured inner mass cells, Proc. Nat'l. Acad. Sci. USA 91(13):6143-7	Cow	1994		Yes
Doetschman et al., Establishment of hamster blastocyst-derived embryonic stem (ES) cells, Dev. Biol. 127: 224-227	Hamster	1988	Yes	
Evans et al., Theriogenology 33: 125-128	Pig, cow	1990	Yes	
Sukoyan et al., Mol. Reprod. Dev. 93: 418-431	Mink	1992	Yes	
Iannaccone et al., Dev. Biol. 163: 288-292	Rat	1994	Yes	

Thus, a person of skill in the art would have recognized that the invention could be practiced with stem cells from mammals other than mice.

7. I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: January 19, 2004

Dr. Jürgen Hescheler

DEVELOPMENTAL BIOLOGY 203, 290–294 (1998)
ARTICLE NO. DB988999

β-Lactamase as a Marker for Gene Expression in Live Zebrafish Embryos

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In this report we describe the development of a sensitive assay for gene expression in zebrafish embryos using β-lactamase as a reporter gene. We show that injection of a green fluorescent substrate for β-lactamase allows the detection of reporter gene expression in live embryos. The β-lactamase enzyme catalyzes the hydrolysis of the substrate, thereby disrupting fluorescence resonance energy transfer from the donor to the acceptor dye in the molecule. As a result, a blue fluorescent product is produced and retained specifically in cells within which the enzyme is expressed. β-Lactamase is therefore suitable for monitoring spatially restricted patterns of gene expression in the early embryo. We suggest that this new reporter system provides a major advancement in sensitivity over the existing methods for monitoring gene expression *in vivo* during early embryogenesis. © 1998 Academic Press

Key Words: zebrafish; β-lactamase; dominant marker.

INTRODUCTION

Monitoring spatially restricted patterns of gene expression in live embryos became widespread when the green fluorescent protein (GFP) was introduced as a reporter gene (Chalfie *et al.*, 1994). GFP can be used for monitoring intracellular protein trafficking, as a dominant marker for transgenesis and cell sorting and, importantly, it can allow the visualization of gene expression patterns in live embryos (Amsterdam *et al.*, 1995; Long *et al.*, 1995; Rizzuto *et al.*, 1995; Takada *et al.*, 1997; Wang *et al.*, 1998; reviewed in Prasher, 1995).

While GFP provides an excellent noninvasive method for monitoring gene expression, the production of signal by this protein is noncatalytic. For this reason, the number of molecules required to overcome the autofluorescence background is relatively high. It was estimated that 10^5–10^6 GFP molecules per cell are required to visualize the protein when it is evenly distributed in the cytosol (Niswender *et al.*, 1995). This low level of sensitivity can result in a time delay between onset of gene expression and the ability to detect accumulated green fluorescent protein (Davis *et al.*, 1995), especially when weak and tightly regulated promoters are used.

Using the FDG substrate for β-galactosidase, it was possible to sort live dissociated *Drosophila* cells that express *lacZ* (Krasnow *et al.*, 1991), and a modification of this method allowed the detection of *lacZ* expression in live zebrafish embryos (Lin *et al.*, 1994). While detection of *lacZ* expression enabled the discrimination between transgenic and nontransgenic fish, false-positive signals were obtained and no resolution at the cellular level was demonstrated (Lin *et al.*, 1994).

To develop a more sensitive and reliable reporter for gene expression, we have examined β-lactamase as a marker for gene expression in live zebrafish embryos. β-Lactamases are very efficient bacterial enzymes for which several colorimetric substrates are known. Recently, a membrane permeable ester derivative of a fluorogenic substrate for β-lactamase was developed which allows the detection of enzyme activity in intact mammalian tissue culture cells (Zlokarnik *et al.*, 1998). The substrate is composed of two fluorophores attached to cephalosporin which brings them close together to allow for efficient fluorescence resonance energy transfer (FRET) (6-chloro-7-hydroxycoumarin was used as the donor and fluorescein as the acceptor fluorophore). When the intact substrate is excited by light at 409

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nm, emission of the acceptor at 520 nm (green) can be observed. β-Lactamase attack splits off the fluorecein moiety from the rest of the molecule resulting in disruption of the FRET and a shift to the emission of the donor at 447 nm wavelength (blue).

Here, we describe experiments using the newly developed fluorescent substrate for β-lactamase in live zebrafish embryos. We show that the substrate we used allows for low levels of gene expression to be monitored, providing a new highly sensitive method for studying early embryogenesis in zebrafish.

MATERIALS AND METHODS

The β-lactamase gene was amplified by PCR from the pBluescript plasmid and was cloned into pBluescript for production of mRNA *in vitro*, or into the pXex vector (Johnson and Krieg, 1994) to be used in DNA injection experiments. The PCR primers used for β-lactamase amplification replace the bacterial signal presequence by codons for Met-Gly. Both the neomycin resistance gene and the GFP gene were cloned into the same vector (pXex) to allow a controlled comparison between the markers. The pXex-GFP construct (pESG) was provided by C.-B. Chien and D. Gilmour.

One-cell-stage embryos were dechorionated using 4 mg/ml pronase in 0.3× Danieau's solution (174 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, 0.18 mM $Ca(NO_3)_2$, 0.15 mM Hepes, pH 7.6). The embryos were washed and microinjected. CCF2 (at a concentration of 0.25, 1, or 2 mM) was injected in a 50 mM KCl solution with mRNA, with plasmid DNA or alone. *In vitro* transcribed mRNA was prepared using the mMessage mMachine (Ambion) and was injected at a concentration of 50 ng/μl. DNA was injected at a final concentration of 10 ng/μl.

The embryos were grown at 25°C and were observed during different stages of development.

CCF2 fluorescence was visualized using the following filter set obtained from Omega Optical: excitation 405 (DF15), dichroic 420 (DRLPO2), and emission 435 (EFLP). The intensity of the 100-W mercury lamp was reduced to 30% and exposure time was reduced to a minimum to avoid bleaching of the fluorescein acceptor. Typically exposures of less than 1 s were required for photographing CCF2-loaded embryos at a 50× magnification using Fujichrome Provia 1600 ASA film. For GFP detection the full intensity of the lamp was used and exposure times for photography were three times longer.

RESULTS AND DISCUSSION

Introduction of a Fluorescent Substrate for β-Lactamase into Zebrafish Embryos

A fluorogenic substrate ester for β-lactamase that is nonpolar enough to cross membranes of mammalian cultured cells was recently developed (Zlokarnik *et al.*, 1998). This substrate molecule can be introduced into cells grown in tissue culture by incubating the cells in an aqueous buffer containing 1 μM substrate ester, CCF2/AM. Introduction of the substrate through the medium is easy and fast, allowing high throughput applications to be carried out. However, dechorionated 1- to 4-cell-stage embryos incubated for 1 h with the highest concentration of the soluble substrate (3 μM) did not take up any detectable amounts of substrate, such that no fluorescence was detected in the embryo. Our attempts to introduce the substrate by increasing the concentration of the dispersing agent Pluronic F127 and by mild sonication did not solve the problem (not shown). We conclude that in contrast to cells grown in tissue culture, cells of zebrafish embryos at the first cleavage stages are inaccessible to the substrate. It is possible that in contrast to cells at later stages of development, cells at the early cleavage stages still possess components of the egg envelope, a multilayer structure that is deposited during oogenesis (Selman *et al.*, 1993) which may not allow substrate ester penetration. Since the substrate is unable to diffuse from cell to cell, application of the substrate through the medium would be ineffective even if cells become permeable for the substrate at later stages.

Because the membrane-permeable ester derivative could not be introduced into early embryos, we decided to microinject the nonesterified CCF2 substrate into early embryos. Indeed, the injected embryos exhibited intense green fluorescence from the time of injection until the tail bud stage (Figs. 1A–1E). Following the tail bud stage, only very low amounts of the substrate could be detected in the embryo. While we do not know by what mechanism the substrate is cleared from the embryo, we have noted that from the initiation of epiboly, accumulation of very light blue fluorescence is observed around the yolk (e.g., in the right embryo in Figs. 1D and 1E) concomitantly with the decrease in green (substrate) and blue (product) fluorescence in the embryo. Hence, under the conditions we used, the β-lactamase–CCF2 marker system can be utilized in zebrafish during presomitic stages only.

Examination of Possible Effects of CCF2 on Zebrafish Embryos Survival

Using CCF2/AM in living mammalian tissue culture cells allows gene expression to be monitored and facilitates cell sorting based on the degree of substrate conversion developed (Zlokarnik *et al.*, 1998). However, the possible toxicity of CCF2 in developing embryos whose survival depends on function of many different cell types remained to be determined. Toward this end, we injected one-cell-stage embryos with the β-lactamase substrate at three concentrations. The results of this experiment are summarized in Table 1. When compared with noninjected fish, injection of 0.25– 2 mM CCF2 does not seem to significantly influence the viability of the embryos as well as of the developing fish. Therefore, the substrate for β-lactamase can be used in fish without affecting cell viability, while potentially allowing visualization of gene expression in live embryos and sorting of live cells.

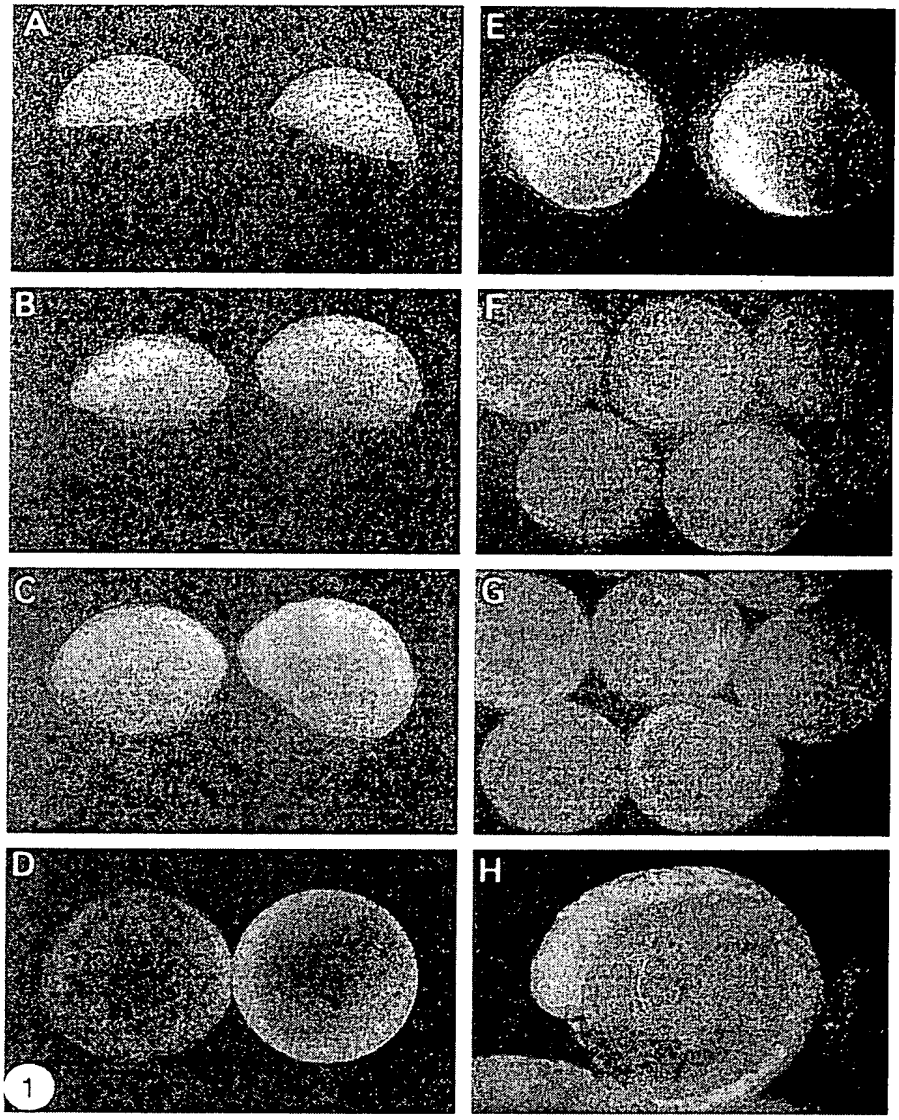

FIG. 1. Rapid detection of β-lactamase gene expression. (A–D) One-cell-stage embryos were injected with 1 mM CCF2 (right embryo) or with 1 mM CCF2 + 10 ng/μl pXex-β-lactamase plasmid DNA (left embryo). Detection with the CCF2 filter set at the oblong stage (3.7 h at 28.5°C) (A), sphere (4 at 28.5°C) (B), 30% epiboly (4 h at 28.5°C) (C), 80% epiboly (8.5 h at 28.5°C) (D), and 10 somites stage (14 h at 28.5°C) (E). (F–H) One cell stage embryos were injected with 10 ng/μl of pXex-mmGFP plasmid and fluorescence was detected using the fluorescein filter set at 50% epiboly (5.3 h at 28.5°C) (F), 80% epiboly (8.5 h at 28.5°C) (G) and at the 10 somites stage (14 h at 28.5°C) (H).

β-Lactamase Can Be Used as a Cell Autonomous Dominant Marker

In order for β-lactamase to serve as a marker for gene expression, both the enzyme and the cleaved product should be confined to the cells that expressed the protein. To test this point we first injected 1-cell-stage embryos with CCF2 and at the 16-cell-stage β-lactamase mRNA was injected to obtain mosaic expression of the marker protein. Indeed, the injected embryos show regions where the substrate was cleaved as well as other regions where the substrate was left intact as indicated by regions of cells showing blue and green fluorescence respectively (Fig. 2A).

To demonstrate the colocalization of the β-lactamase RNA and the blue signal, we used rhodamine labeled dextran to trace the cells that were injected with the RNA. Embryos preloaded with CCF2 were coinjected with rhodamine dextran and β-lactamase mRNA at the 64-cell stage. At 30% epiboly stage, the yolk was removed and the

TABLE 1
Survival of CCF2-Injected Zebrafish

CCF2 concentration	Survival rate 24 h	2 months
Noninjected	0.99 ± 0.01	0.71 ± 0.03
0.25 mM	0.96 ± 0.06	0.80 ± 0.11
1 mM	0.98 ± 0.04	0.72 ± 0.07
2 mM	0.86 ± 0.18	0.59 ± 0.19

Note. Fish were injected with CCF2 at the concentrations indicated in the table. The fraction of the fish that survived was determined after 24 h and after 2 months. The results were obtained in 3 independent experiments on a total of 106 embryos (0.25 mM CCF2), 134 embryos (1 mM CCF2), and 144 embryos (2 mM CCF2).

embryos were visualized using fluorescent filters for rhodamine or with filters for CCF2 detection. Only cells showing rhodamine fluorescence also show blue fluorescence indicative of β-lactamase activity (Figs. 2B and 2C). We therefore conclude that spatially restricted gene expression patterns can be identified using β-lactamase as a marker in early embryos.

β-Lactamase Is an Extremely Sensitive Marker for Gene Expression in Live Zebrafish Embryos

To compare the sensitivity of the β-lactamase system with that of GFP, we tested our ability to detect gene expression in fish injected with either β-lactamase or GFP expression constructs. For this purpose, the *β-lactamase* gene, the *mmGFP* gene which is a version of GFP that exhibits improved stability and fast formation of active chromophore (Siemering *et al.*, 1996), and the neomycin gene (as a negative control) were cloned downstream of the *Xenopus* EF1α promoter (Johnson and Krieg, 1994) (the GFP construct pESG was kindly provided by C.-B. Chien). CCF2 and low concentrations of the plasmids (10 ng/μl) were injected into one-cell-stage embryos. The developing embryos were monitored for green fluorescence and for conversion of the fluorogenic β-lactamase substrate from green to blue fluorescence. For detection of the CCF2 substrate we used only 30% of the light intensity provided by a 100-W mercury lamp. Nevertheless, conversion of the β-lactamase substrate was detected much earlier than the fluorescence generated by GFP for which we used the full power of the lamp (Figs. 1B and 1F). Even under these conditions, the intensity of the signal was stronger for the β-lactamase as manifested by a significantly shorter exposure time (i.e., 33%) required for photographing the embryos. Blue fluorescence was visible in embryos immediatly following the initiation of zygotic transcription at midblastula transition (MBT) (Kane and Kimmel, 1993), just before the sphere stage. β-Lactamase activity was frequently detected first at a specific part of the embryo (Fig. 1B), presumably close to the injection point resulting in a gradient of DNA concentration in the embryo. The initial localized signal has spread rapidly and, in most cases, conversion of substrate was detected throughout the embryo by 30% epiboly (Fig. 1C). No conversion of substrate from green fluorescent to blue fluorescent was observed in noninjected embryos (Figs. 1A–1E) and neither in embryos

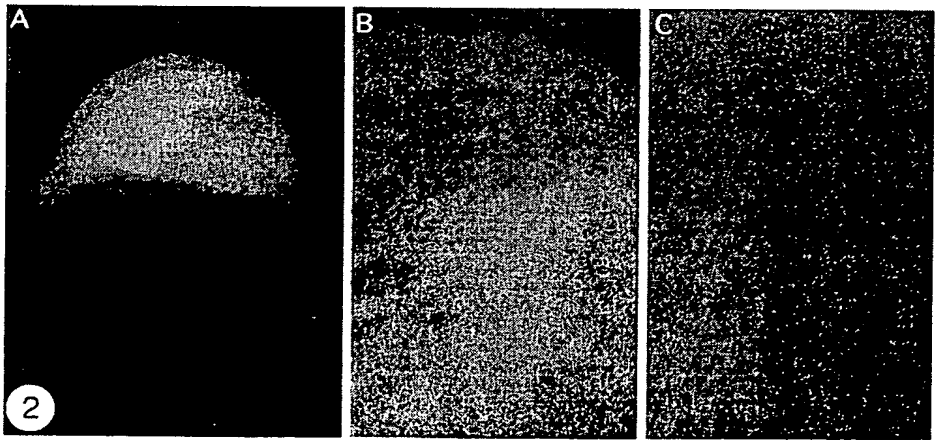

FIG. 2. Cell-autonomous detection of the β-lactamase marker. (A) One-cell-stage embryos were injected with CCF2 and later at the 16-cell stage one of the marginal blastomeres was injected with β-lactamase mRNA. At this stage the cytoplasm of the marginal blastomeres is not separated allowing the mRNA to diffuse and to be translated in more than 1/16 of the cells. (B, C) One-cell-stage embryos were injected with CCF2 and later at the 64-cell stage one of the blastomeres was coinjected with β-lactamase mRNA and with rhodamine dextran as a lineage marker. At 30% epiboly the yolk was removed and a flat preparation of the embryo was visualized at a high magnification using the CCF2 filter set (B) or with a filter set for rhodamine detection (C).

injected with a DNA construct expressing the neomycin resistance gene under the control of the *Xenopus* EF1α promoter (not shown). The accumulating light blue fluorescence around the yolk (control embryos in Figs. 1D and 1E) is significantly different from the positive blue signal allowing reliable discrimination between β-lactamase-injected and noninjected embryos even after the substrate and the product are cleared from the embryo proper (Fig. 1E).

At the early stages of embryogenesis, no GFP fluorescence could be detected when the same amount of plasmid was injected. Weak GFP expression could be first observed in small clusters just prior to the shield stage, but it was not until 70% epiboly that a significant number of cells (20–30%) showed green fluorescence. Expression of GFP in the majority of the cells at the level detected by the β-lactamase at 30% epibol, was achieved only around the 90% epiboly. Standardized for development at 28.5°C, we found that using β-lactamase as a marker, we could detect gene expression in cells that receive low number of DNA molecules 4–5 h before those cells would exhibit detectable cytoplasmic GFP expression.

As mentioned above, the substrate is cleared from the embryo after the completion of epiboly, making β-lactamase–CCF2 a useful system for monitoring gene expression before somitogenesis. In contrast, GFP can be used at later stages, depending upon the translucency of the tissue (Fig. 1H). Thus, β-lactamase can be the marker of choice either when low levels of gene expression are monitored or when rapid detection is required in early embryos. The use of the β-lactamase–CCF2 system at later stages is not possible by applying the method described here. Therefore, to detect gene expression in live embryos at later stages of zebrafish embryos, GFP remains the marker of choice.

CONCLUSIONS

We have demonstrated that the CCF2–β-lactamase system can be used in live embryos to monitor gene expression. The system is unique in that it is highly sensitive, yet allows high-resolution detection of gene expression patterns without compromising viability. While the experiments described in this report were carried out using zebrafish embryos, this system is likely to be applicable for embryos of other organisms such as *Drosophila* or *Xenopus*.

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Accepted June 26, 1998

Dear Bernd and Eugene,

Eugen>promoters with GFP to monitor the ES-c lls differentiating to cardiac
Eugen>on s. Shur ,in this course we should replace the actin promoter in
Eugen>pCX-xGFP for appropriate ones. Obviously, we will preliminary
Eugen>check the pCX-xGFP expression itself in ES-cells.

I attached pCX-GFP map and sequence. They are basically the same to pCX-h-x-GFP. We are not allowed to disclose the sequence of h-x-GFP. Our construct may not work nicely in ES cells from our experience. In order to check the high expression of h-x-GFP you had better use other cell lines.
In the map, I wrote "FITC filters" are not good. However, this is for wild type GFP. h-x-GFP works better with FITC filters (excitation 488).

Good luck!

Masaru

I'll be in Norway in the next 10 days.

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PH'0S)#p%CACTBf9$69P,)'9a)'pb)'pbIAY8I@PQC@acCAeLC'B0,h0PG'0YH@Y
MEfa[FL"hD'9bCAX[Ff9dBfejDf0[E'pb)'GPG#![BhCMEAPV)'9iBfJJC'9QIAX
[BhCMEAPVHc%JFh9L)$3J-5"bEfaX)$0I-b"TEQ4PH#"KC'3JEQ9R)'4eF#!`)'a
dHh"[F#!`I@PQ)$-J-5"bEfaXIA*PF'9KG#"cCA4bCf*MEfa[FL"`Eh"pBQ4Q)(e
TCQ9XFf80,h0dChYTFd4PGQPMC80[E'pb)(YMCL"MB5![Bh-JE'pKC#"cCA4cBh*
PC@iJFf9dCh*KHAelF'p`I@PQC@acCAeLC'B0,h0dFQGLHfPc4'9fD@0P3fpXEh)

K. Fleischmann>x-GFP version and alternatively in the case it didn't work so
K. Fleischmann>well for us with the other ones we would be very
appreciative indeed.

I think if x-GFP wouldn't work, no other version would work. This is very bright. If it doesn't work and if you really need to proceed to use GFP, you have to try another promoter.

Sincerely,

Masaru

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